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Time Kill Assay Evaluation of *Piper guineense* Leaf and Seed Extracts against Enteric Pathogen

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ABSTRACT

Piper guineense is a vital herb in use for the treatment of infections in folklore medicine. It's also used as an additive to food to enhance the taste during food processing. Most people do not understand its medicinal usages and value. The aim of this study was to evaluate the antimicrobial susceptibility and the time kill activity of *Piper guineense* leaf and seed against some enteric pathogen and determine the proximate *and* phytochemical constituents that support the clinical use. The leaves and seeds of *Piper guineense* were dried under shades and ground to a fine powder, screened for its proximate and phytochemical constituents using standard methods. The antimicrobial susceptibility was done using agar well diffusion method; serial doubling dilution (MIC) and time kill assay activity. The pathogens used were *Shigella dysenteriae. Escherichia coli, Staphylococcus aureus* and *Salmonella typhi*. The phytochemical analysis showed that it contained alkaloids, flavonoids, tannins and saponin. The in-vitro antimicrobial activity of *Piper guineense* against enteric pathogen showed that the extracts are sensitive to the microorganisms tested. The most sensitive was aqueous extract of the seeds on *Salmonella typhi* with mean zone of inhibition of 35.0±0.3mm.The time kill assay showed reduction in number of viable cells cfu/ml on incubation. At MIC there was a reduction between 6-8hrs, while at 2 × MIC reduction occurs within 3- 4hours. This research reveals that *P. guineense* can serve as a potential antimicrobial in the treatment of infections based on its antimicrobial activity.

Keywords: *Piper guineense,* Antimicrobial, Proximate, Phytochemical Methanol, In-vitro

Introduction

Herbal plants, usage in the treatment of diseases and infections is as old as man. Researchers are interested in studying herbal plants that are under-researched to spot the compounds in them that are potent and find the scientific backup of such plants as used in traditional herbal medicine. Generally, the major objective is to make a synthetic copy of the compound that can be readily produced in the laboratory and incorporated into therapeutic preparations.[1]*Piper guineense* is a plant species belonging to the genius piper and family *Piperaceae.* It's indigenous to West Africa.[2]It is known as black pepper in English, Uziza in Igbo, Iyere in Yoruba, Masoro in Hausa, Sorowisa by the Ghanaians and poivrie in French. The seeds and leaves are both used as spices in the preparation of various cuisines. *Piper guineense* contains chemical compounds that have anti-microbial effects.[2] In folklore medicine, the seed is given to women after childbirth to help remove other remains from the womb.[3]The fruits and leaves are used for treating stomach aches, nausea, worms expelling, rheumatism, malaria, tonsillitis and to aid wound healing.[4] The leaf and seed extracts act as depolarizers for neuromuscular activity.[5] The leaf and seed have been used for relief of cough, combating respiratory diseases, serving as anti-microbial, anti-inflammation and aiding digestion.[3, 4]

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The seed extract is taken, to easy childbirth and haemorrhoids.[2] Mixing of seeds, leaves and roots can be used for preparing antibacterial agents for the management of infectious diseases.[6]
The time-kill assay gives an in-depth understanding of the in vitro interactions between microbes and antimicrobial agent over a given period. It is also used to determine the bacteriostatic and bactericidal concentrations. It monitors the effect of various concentrations over a given time against microorganisms.[7]
Shigella dysenteriae.Staphylococcus aureus, Escherichia coli and,*Salmonella typhi* are enteric pathogens implicated in food-borne illnesses as a result of ingestion of contaminated food and water causing diarrhea, dysentery. Enteric pathogens causes a health burden on humans leading to increase morbidity and mortality creating economic loss in most part of the world and is more severe in children and thoseimmunocompromised.[8] Factors such as antibiotic resistance, globalization and, environmental changes have encouraged the spread.[9]The virulence factor of enteric pathogens is in their ability to secrete macro molecular syringes that translocate the effector proteins to the host cytosol[10] Releasing of toxins through a toxin delivery system is vital for the persistence, pathogenesis and survival of the enteric pathogens. Treatment of infection caused by these bacteria cannot be eliminated using most modern antibiotic therapies because some of the antibiotics cause serious complications from antibiotic-induced endotoxin, ineffectiveness and, expensive.[10] Therefore, newer therapies that will have proven efficacy, low toxicity and readily available should be sorted for.

Materials and Method

Collection of Plant material
Fresh leaves and seeds of *P. guineense* were purchased from New Market a major market in Enugu State Nigeria in August 2022. The plant parts was identified, authenticated and given a voucher specimen

number UNH/ 09/0229D by a taxonomist in Department of Plant Science and Biotechnology, University of Nigeria Nsukka. A voucher specimen was deposited at the herbarium. The leaves and seeds were clean using distilled water and spread under shade and allowed at room temperature to dry. The dried seeds and leaves were grounded to fine powder using electronic blender and stored in sterile air tight bottle till ready for use.

Preparation of plant for extraction
The leaves and seeds (500g each) of *Piper guineense* was extracted by cold maceration in 95% aqueous methanol (2. 5L) for 48 hours with constant agitation. The extracts were dried in vacuo at reduced temperature and pressure to obtain the dried extracts. The extracts were separately dissolved in 500ml of 10% methanol and partitioned successfully using n-hexane (4×500ml) and ethyl acetate 6 × 500ml) to obtain the solvent fractions.

Sterility of the extracts
This was done by culturing them on sterile Mueller Hinton Agar for 24hours and then stored in sterile amber bottles at 4^{o}C.
Proximate Analysis
The leaf and seed samples were evaluated for protein, ash fiber, moisture and dry matter contents using standard method of .[11] Mineral Analysis done were Potassium, Copper, Magnesium, Iron, Sodium, Calcium and Zinc using atomic absorption spectrometric method.

Phytochemical Screening
The phytochemical constituents of extracts determine using standard method.[12]

Isolation of Test Organism
The bacteria isolates used for this study were clinical bacteria isolates (*Escherichia coli Shigella dysenteriae* , *Staphylococcus aureus* and *Salmonella typhi)* were obtained from Department of Microbiology University of Nigeria Teaching Hospital Ituku-Ozalla. These microorganisms were preserved on Mueller-Hinton agar (MHA).The test isolates were vortexed (using EN-04726-01 by Cole-Parmer, United states) vigorously and adjusted to 0.5 McFarland standard before use

Media preparation
The media, Mueller-Hinton agar (MHA) were prepared according to the manufacturer's instructions. About 20mL of the sterilized medium was cooled to about 50^{0}c and was aseptically poured into a ninety mm diameter Petri dish and allowed to solidify. Sterility of the prepared media was assessed by incubation in an incubator (DNP-9052-1A by Medified equipment and scientific limited, China) by randomly selecting 3 plates and incubating them at 37°C for 24 hours.

Determination of Antimicrobial properties of the extract
The antimicrobial properties of extracts and fractions were evaluated using agar diffusion method.[13] Briefly, sterile swab sticks were dipped into the already standardized cell suspension, the swab was pressed against the test tube to remove excess fluid and was evenly rolled over the whole media surfaces by rotating the plate to obtain an even inoculum distribution on the agar plate and allow to dry for 10 mins. A sterile metal cork borer, with a diameter of 6 mm, was used to create wells in the MHA. Each well was filled with 0.5 mL of each extracts at 200mg/mL and allowed to stand for 20 minutes so that the extract can diffuse into the agar. Ciprofloxacin (0.05mg/mL) was used to serve as the positive controls while the negative control was Dimethyl Sulphuroxide. The inoculated plates were incubated at 37ºC for 24 h. The extract and fractions was tested against all the bacteria isolates and each was done in triplicate against each organism. After incubation, inhibition zone diameter (IZD) of each well was measured in triplicate and recorded to the nearest millimeter and the mean readings were calculated.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)
The MIC were assessed by adopting the method used by CLSI[14] For each of the extract 5 mL of Muller Hinton agar broth was placed in test tubes and 1mL of each extract and fractions introduced into the first tube and serial dilution of the each extract and fraction is undertaken to reduce the concentration in the broth serially (200, 100, 50, 25 12.5, 6.25 mg/mL). A standard inoculate (0.1 mL) of the test organism (adjusted to 0.5 McFarlands standard) was introduced into each of the test tubes. All the tubes were incubated overnight at 35 - 37°C. The MIC is the broth containing the least extract and fraction concentration, which showed no turbidity (evidence of no growth) in the tube. The tubes without turbidity was cultured on MHA plate and incubated to determine the MBC. The Minimum bactericidal concentration is the least extract concentration that did not show any visible growth on the media.

Time kill assay
The MIC concentration that was obtained in the bacteriostatic assay was used to determine the time-kill assay. This was assessed at ½ × MIC, 1×MIC and 2 ×MIC of each test isolates obtained. A 0.1mL of aliquot was removed from the broth at 0, 2, 4, 6, 8, 10 and 12 h and a 1: 10 dilution of it made. It is then platted onto MHA and incubated at 37^{O}C overnight. The resulting colonies were calculated and expressed as cfu/mL[15] The time kill assay curve was plotted between the time of incubation and logarithmic number of cfu/m. A bactericidal activity is said to occur when there is $3\log_{10}$ fold reduction when compared to the initial inoculums which is equivalent to 99.9% death of viable cells.

Statistical Analysis
The results were expressed as Mean ±SE n=3. The significance level was set at P $\leq$0.05. Data obtained were statistically analyzed using one-way Analysis of Variance ANOVA.
Statistical package for social Sciences (SPSS) version 22 was used.

Result and Discussion

Proximate analysis
P. guineense has been found to contain nutritional components. Proximate and mineral analysis of the seed and leaf as shown in Table 1 reveal that it comprises fiber, proteins, carbohydrates, fat, mineral and, vitamin and agrees with previously reported works.[16-18] The mineral analysis showed that *P. guineense* contained Fe, Zn, Mg, Ca, Na and K. Minerals such as sodium, calcium and potassium which is vital for physical and mental development and very useful in the formation of bones, tissues and teeth muscles.[3] Iron is essential for optimal performance of the nervous system, haemoglobin formation, and help in protein oxidation, fats and carbohydrate.
Phytochemicals
Phytochemical analysis of *P. guineese* showed the presence of tannins, alkaloids, glycosides, flavonoids, and saponins. Table 2. The presence of these phytochemicals reveals its vital role in traditional medicine. The high amount of alkaloids seen in the seed and leaf is an important pointer to its significance in folklore medicine thus; alkaloids are rated as the most resourceful therapeutically plant material.[19]The report of [20]shows similar plant chemicals from the leaf extracts of *P. guineense.* Alkaloids help in the elimination of infectious diseases because of their high antimicrobial properties which makes them mostly used during drug production. Alkaloids cause cell death by interfering with the cell component.[21] Tannin helps in protein denaturation in bacteria leading to cell death. [22] Flavonoids are lipophilic agents, its enzymatic activity causes inhibition of bacteria cell membranes and cell wall formation by binding with the bacteria cell wall[23]. Saponin creates a cytotoxic effect by inducing the entry of toxic material into the cell.[22]

Antibacterial Activity
The in-vitro anti-bacterial activity of *P. guineense* leaf and seed extracts and fractions against enteric pathogens as presented in Table 3 showed different zones of inhibition for the bacteria. This is in line with the works of [24-25] who reported an active inhibitory effect of the seed and leaf of *P. guineense* towards both gram positive and negative

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organisms. The highest inhibition obtained was recorded for the seed extract was the aqueous seed extract with 35.0±0.7mm zone of inhibition against *Salmonella typhi* and *S. aureus* which was statistically different (p<0.5) and followed by ethyl acetate 32.0±0.5mm against *E.coli* and the least was n-Hexane 27.0±0.2mm against *S. aureus.* The highest zone of inhibition for the leaf extract was methanol 32.0±0.0mm which was statistically different (p<0.5) against *Salmonella typhi* followed by aqueous extract 30.0±0.8mm against *E.coli* and *Salmonella typhi* while the least was n-Hexane 27.0±0.5mm against *S. aureus* and *E.coli*

P. guineense possesses a potent in vitro activity against the isolates tested. Although the standard antibiotics Ciprofloxacin had a better zone of inhibition that had a statistically significant difference (p<0.5) which could be as a result of the use of more refined and pure compounds used in the preparation

Table 1: Proximate and mineral analysis of *Piper guineense* seed and leaf (g/100g)

Parameters	Seed	Leaf
Fiber	9.72	11.5
Moisture	12.34	10.2
Fat	7.72	4.6
Protein	10.14	23.8
Carbohydrates	51.27	42.1
Mineral	Mg/100g	
Fe	0.37	8.92
Mg	0.092	510
Zn	0.208	5.78
K	0.97	580
Ca	0.66	294
Na	0.52	35.0

Table 2: Phytochemical constituents of *Piper guineene*

Phytochemicals	Leaves	Seeds
Alkaloids	+	+
Flavonoids	+	+
Saponins	+	+
Tannins	+	-
Glycoside	+	+

The result obtained from this study showed a statistically significant different (P<0.5) between the two plant parts of *P. guineense* (leaf and seed) depending on the solvent used and its activity against all the isolates tested. Comparing the mean zone of inhibition of *P. guineense* leaf methanol extract had better antibacterial activity with zone of inhibition 32±0.0 against *Salmonella typhi* which was statistically significant different (P<0.5) than other solvents used while for the seed the aqueous seed extract has a more antibacterial activity with highest zone of inhibition 35.0±0.7mm against *Salmonella typhi* and *S. aureus* which was statistically different (P<0.5). Comparing the solvents used, aqueous and methanol had better zones of inhibition against all isolate tested and it shows a significant difference (P<0.5)

The aqueous extracts of the leaf and seed against the bacteria isolates showed a high zone of inhibition towards all the extracts this could be as a result of the fact that most bioactive compounds in *P. guineense* are soluble in water. This is similar to the works of.[25] This also justifies its indigenous use in the eastern part of Nigeria where its aqueous extract and is given to women in labour to ease childbirth and after delivery to prevent the colonization of bacteria.[3] It's aqueous exact is also used in treating stomach aches and haemorrhoids.[25] This also shows that solvents used during extraction have a vital role to play.[26].

MIC and MBC

The lowest concentration of seeds and leaves extracts that have no visible growth (turbidity) after overnight incubation upon visual observation was regarded as the MIC value. The methanol leaf extract of *P. guineense* as shown in Table 4 inhibited *E. coli* and *Shigella dysenteriae* at 12.5 mg/ML and *S. aureus* and *Salmonella typhi* at 25mg/mL while the methanol seed inhibited the growth of *E. coli* at 12.5mg/ML and other isolates at 25mg/ml. The aqueous, ethyl acetate and n-hexane leaves and seeds of *P. guineense* inhibited the growth of all the test isolates at12.5mg/ml, 25mg/ml and 50mg/ml respectively.

The MBC is the concentration at which cell death occurs and is very important in therapeutic use. The MBC of *P. guineense* leaf extracts ranges between 25 mg/mL to 50 mg/mL as shown in Table 4. The methanol and ethyl acetate leaf extract inhibited *E. coli* and *Shigella dysenteriae* at 25 mg/ML and *S. aureus* and *Salmonella typhi* at 50mg/mL. The n-hexane leaf extract eliminated *Shigella dysenteriae* at 50mg/ML and the other bacteria isolates at 100mg/ml. The aqueous leaf extract eliminated all the test isolates at 25mg/MlL. The MBC of the *P. guineense* seed extracts ranges between 25 -100mg/ML. The methanol seed extract eliminated *E. coli* at 25mg/ML and other isolates at 50mg/ML. The aqueous, ethyl acetate and n-hexane eliminated all the test isolates at 25mg/ml, 50mg/ml and 100mg/ml respectively Methanol and aqueous extracts both leaf and seed of *P. guineense* had a better MBC value than ethyl acetate and n-hexane against the isolates tested. The MBC value ranges between 25 -100 mg/mL. It shows that *P. guineense* can be of use in treating infectious conditions caused by the isolates tested. .

Time kill assay

Time-kill assay activity was done for 12 h with methanol crude extracts at ½ × MIC, 1×MIC and 2 × MIC concentrations respectively of each test isolate. The time-kill curve shows a great decrease in the number of viable cells. At 2×MIC, it took 4 hours for double MIC concentration of *P. guineense* methanol seed extract as shown in Figure 1 to cause 99.9% cell death for *S. aureus,* less than 5hours for *Salmonella typhi, E. coli* and *Shigella dysenteriae.* At 1×MIC, the methanol seed extract inhibited growth of all the isolates within 8-12hours. The highest reduction recorded at 1×MIC was against *S. aureus* and *Salmonella typhi* at 6hours while *E. coli* and *Shigella dysenteriae* reduction was at 12hours. At ½ × MIC, there was a continuous increase in the number of viable cells with an increase in time because the concentration was not able to cause any inhibitory effect (either bacteriostatic or bactericidal) on the isolates.

The *P. guineense* methanol leaf extract as shown in Figure 2, at 2×MIC caused 99.9% cell death of all tested isolates within in less than 6hours of incubation. The highest reduction recorded at 2×MIC was against *S. aureus* at 4hours of incubation. At 1×MIC a great reduction in the number of viable cells was seen at 12hours of incubation for all the isolates tested. At ½ × MIC, there was a continuous increase in the number of viable cells with an increase in time because the concentration was not able to have any either bacteriostatic or bactericidal effect on the isolates. FIG: 2. The time-killing assay shows the time-dependence of the extract concentrations to completely inhibit the bacteria growth. The effective antimicrobial ability of *P. guineense* can be credited to the vital phytochemical constituents in the plant. The efficacy of the leaf and seed methanol extracts of *P. guineense* was time and concentration-dependent presenting an active time-kill activity on the isolates tested. The in-vitro activity of the extracts gives an understanding of the effectiveness of the plant as an alternative active therapy in treating infections caused by enteric pathogens validating its indigenous use in ethno-medicine. Furthermore, understanding the basic chemical component and isolation of the bioactive constituents in *P. guineense* will create an opportunity for the synthesis of new and effective antibacterial agents

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Table 3: Mean ± SE Zone of inhibition of *Piper guineense* extract and fractions at (200mg/mL) on enteric pathogens

	Leaves extract (mm)				Seeds extract (mm)				Positive control
Bacteria isolates	**Methanol**	**Ethyl acetate**	**n-hexane**	**Aqueous**	**Methanol**	**Ethyl acetate**	**n-hexane**	**Aqueous**	**Ciprofloxacin 0.50mg/ml**
S. aureus	25.0 ± 0.2	25.0 ± 0.4	27.0 ± 0.1	29.0 ± 0.2	28.0 ± 0.1	30.0 ± 0.2	27.0 ± 0.2	35.0 ± 0.7	39.0 ± 0.0
E. coli	30.0 ± 0.1	28.0 ± 0.6	27.0 ± 0.5	30.0 ± 0.4	30.0 ± 0.5	32.0 ± 0.5	30.0 ± 0.6	32.0 ± 0.3	37.0 ± 0.0
Salmonella typhi	32.0 ± 0.0	28.0 ± 0.3	28.0 ± 0.9	30.0 ± 0.8	30.0 ± 0.7	30.0 ± 0.3	28.0 ± 0.8	35.0 ± 0.3	39.00 ± 0.0
Shigella dysenteriae	30.0 ± 0.4	27.0 ± 0.7	29.0 ± 0.3	28.0 ± 0.25	32.0 ± 0.1	30.0 ± 0.4	29.0 ± 0.2	33.0 ± 0.8	36.00 ± 0.0

Table 4: MIC AND MBC of *Piper guineense* Extracts on enteric pathogens

	Leaf extract and fractions (mg/mL)								Seeds extract and fractions (mg/mL)							
Bacteria isolates	**Methanol**		**Ethyl acetate**		**n-hexane**		**Aqueous**		**Methanol**		**Ethyl acetate**		**n-hexane**		**Aqueous**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
S. aureus	25.0	50.0	25.0	50.0	50.0	100.0	12.5	25.0	25.0	50.0	25.0	50.0	50.0	100.0	12.5	25.0
E. coli	12.5	25.0	25.0	25.0	50.0	100.0	12.5	25.0	12.5	25.0	25.0	50.0	50.0	100.0	12.5	25.0
Salmonella typhi	25.0	50.0	25.0	50.0	50.0	100.0	12.5	25.0	25.0	50.0	25.0	50.0	50.0	100.0	12.5	25.0
Shigella dysenteriae	12.5	25.0	25.0	25.0	50.0	50.0	12.5	25.0	25.0	50.0	25.0	50.0	50.0	50.0	12.5	25.0

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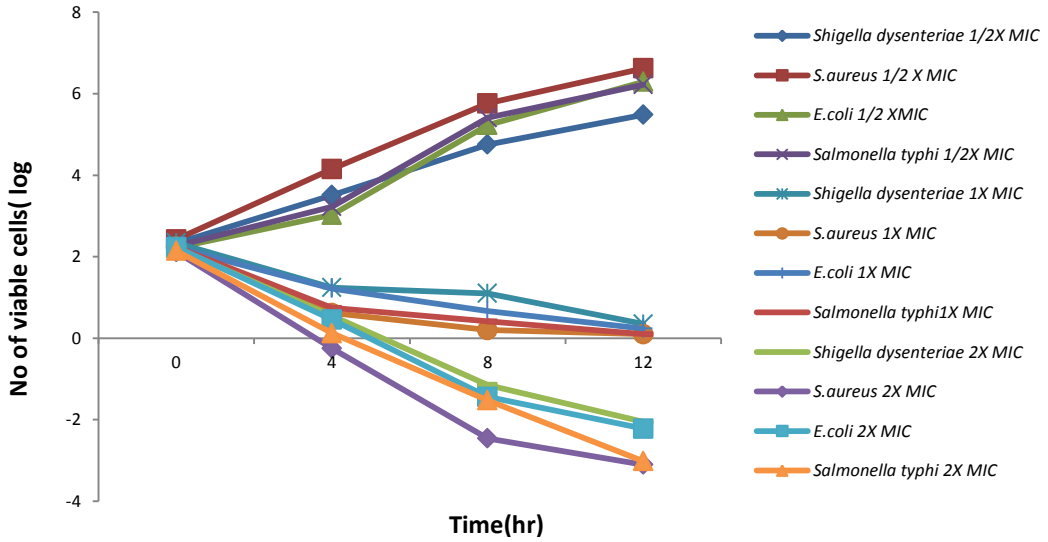

Figure1: In-vitro time kill assay of methanol extract of piper guineense seed

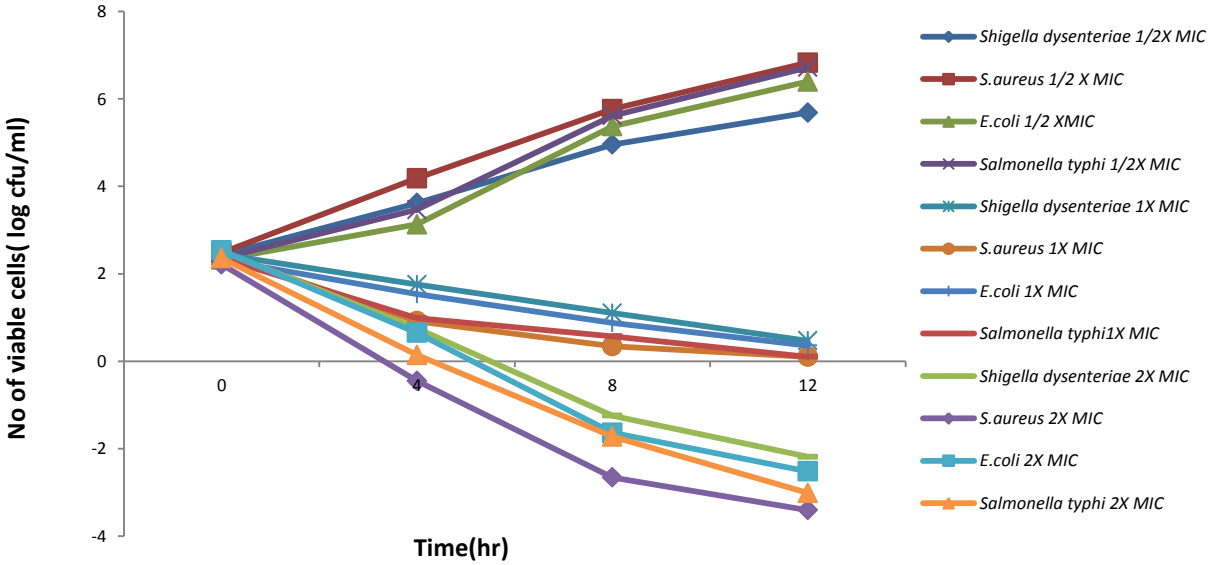

Figure 2: In-vitro time kill assay of methanol extract of piper guineense leaf

Conclusion

The abundant and rich plant nutrients and phytochemicals in the leaves and seeds of *P. guineese*, add up to its importance as a priceless medicinal source to be employed in the treatment of infectious diseases and have been proven in both in-vitro and in-vivo scientific research. The methanol and aqueous extract have a more inhibitory activity than n-hexane and this justifies its use by traditional healers. Further studies need to be done on the toxicity of the plant and on methods of purification and isolation of only the important and active constituents.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.\

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